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ORIGINAL ARTICLE

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Identification of the proto-oncogene c-fos in areas of periprosthetic osteolysis around loosened hip prostheses

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Abstract Expression of the proto-oncogene c-fos was investigated by in situ hybridization and a study of the immunohistochemistry of the interfacial membranes surrounding the femoral component of failed cementless total hip arthroplasty (THA). The values of the proto-oncogene c-fos gene and protein were high in these interfacial membranes. High expression of the c-fos gene and protein was observed in the macrophage-like cells and fibroblast-like cells. However, there was little expression in multinucleated giant cells. We demonstrated the significance of the proto-oncogene c-fos in interfacial membranes – the first report of c-fos in total joint replacement failure.

Key words Proto-oncogene c-fos · Interfacial membrane · Osteolysis

Introduction

Bone resorption around loosened hip prostheses is assumed to involve the production of cytokines by activated macrophages. Nonspecific chronic inflammatory reactions have been found in interfacial membranes obtained from the junction of the prosthetic device and its surrounding bone.

The interfacial membrane is thought to be involved in periprosthetic osteolysis leading to aseptic loosening. Human interfacial membranes contain large numbers of macrophages, fibroblasts, and multinucleated giant cells, many of which have phagocytosed wear debris. Phagocytosis of wear particles is held responsible for the increased production of cytokines, such as interleukin (IL)-1, IL-6, and tumor necrosis factor-alpha (TNF-α).[1,2] The combined actions of such mediators is thought to cause the localized osteolysis that accompanies aseptic loosening. Despite the popularity of this hypothesis, the key mediators have not been identified and the mechanisms controlling chronic inflammation are unclear.

Since synovial cells are characteristic of the interfacial membranes, it appears that growth and osteoclasia caused by the production and secretion of proteolytic enzymes are involved in the process.[3] The features of growth and histolysis seen in such inflamed synovial tissue are similar to those of rheumatoid arthritis. Recently, it was reported that the growth of synovial cells and the production and secretion of proteolytic enzymes such as stromelysin and collagenase in rheumatoid arthritis are strongly influenced by proto-oncogenes.[4,5] Osteosarcoma occurs in transgenic mice with over-expression of the c-fos oncogene,[6,7] while osteopetrosis occurs in the c-fos gene targeting mice.[8,9] This suggests that the c-fos proto-oncogene plays an important role in the induction and differentiation of osteoclasts.

Accordingly, we assumed that this proto-oncogene was involved in the persistent chronic inflammation within interfacial membranes and the periprosthetic osteolysis observed in patients with loosened hip prostheses. We therefore examined the expression and localization of the proto-oncogene c-fos within interfacial membranes using in situ hybridization, and performed an immunohistological study of the localization of the c-fos protein.

Materials and methods

Patients and specimens

Eleven interfacial membranes from 11 patients undergoing revision total hip arthroplasty for aseptic loosening were obtained at our institution between March and September 1997. All 11 patients exhibited osteolysis around the femoral component (Fig. 1). Serial radiographs were compared, according to the zones described by Gruen et al.[10]

Anteroposterior radiographs showed the following distribution of focal osteolysis: multifocal in four cases, proximal (Gruen zones 1,7) in five cases, and distal (Gruen zones 3,5) in two cases. The patients comprised six women and five men, ranging from 53 to 73 years old, with a mean age of 61.6

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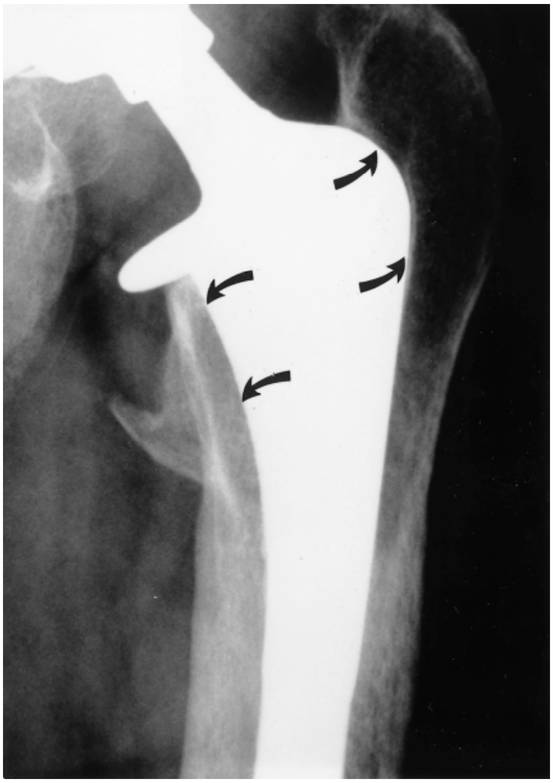

Fig. 1. Focal osteolysis (*arrows*) is apparent around a Harris–Galante femoral component

± 8.2 (SD) years. The time elapsed before revision, for all components involved, ranged from 41 to 73 months, with a mean of 59.8 ± 14.9 (SD) months. The primary diagnosis that led to the first total hip arthroplasty was osteoarthritis in all patients. All of the failed cementless femoral components were made of titanium alloy: five were Harris–Galante (Zimmer, Warsaw, IN, USA), and six were Omnifit stems (Osteonics, Allendale, NJ, USA). The indication for revision surgery was pain and radiographic evidence of loosening in all cases. Normal synovial tissue was retrieved from undiseased joints of patients undergoing above-the-knee amputation and was used as a negative control.

Tissue for RNA analysis was frozen and stored at −80°C until used. Tissue for histological examination was fixed in 4% buffered paraformaldehyde and embedded in paraffin. Serial 5-μm sections were cut from each block, and one slide was stained with hematoxylin and eosin for histological examination. The sections for the in situ hybridization studies were bound to aminopropyltriethoxysiline-coated microscope slides and incubated at 50°C for 2h.

Preparation of cDNA probes

Isolation of total cellular RNA

Cellular RNA was prepared from the interfacial membranes by the guanidinium thiocyanate–phenol–chloroform extraction method.[11]

Reverse transcriptation of RNA

RNA samples containing 10μg total cellular RNA, 50mM Tris-HCl pH 8.3, 75mM KCl, 0.5mM $MgCl_2$, 10mM dithiothreitol, dNTP (0.5mM of each: dATP, dCTP, dGTP, dTTP), 20U RNase inhibitor, 100pM random hexamer (TaKaRa, Tokyo, Japan), and 200U Moloney murine leukemia virus reverse transcriptase (Gibco, BRL, Life Technologies, Inc., Gaitherburg, MD, USA), in a final volume of 20ml, were transcribed by incubation at 37°C for 60min. The samples were heated at 94°C for 5min to terminate the reaction, and then stored at −20°C until used.

Preparation of oligonucleotide primers. Oligonucleotide primers were constructed from the published cDNA sequence of human c-fos cDNA. The following primers were made by TaKaRa (Tokyo, Japan). The sequences of the human c-fos primers were 5′-GACAGACCAACTAGAA GATGA-3′ (coding sense), corresponding to bases 2090–2110 of the cloned full-length sequence, and 5′-GTGGT CACCTGTACTCCCAG-3′ (anticoding sense), corresponding to bases 2565–2584. The predicted size of the amplified c-fos product was 495bp.

Amplification of cDNA

Each reverse transcription mixture was diluted 1:5 in RNase-free water, and 10ml was then transferred to fresh tubes for amplification. Each sample contained the coding sense and antisense primers (200mM of each primer) spanning the given sequences for amplification, 200mM each of digoxigenin-labelled dNTP (dATP, dCTP, dGTP, dTTP), 50mM KCl, 10mM Tris-HCl pH 8.3, 10mM $MgCl_2$, and 2.5U Ex Taq DNA polymerase (TaKaRa, Tokyo, Japan), in a final volume of 100μl. The amplification profiles consisted of denaturation at 94°C for 1.5min, annealing at 58°C for 1min, and extension at 72°C for 1min. Negative control reactions without a template were routinely included in polymerase chain reaction (PCR) amplifications with both primer sets. In preliminary experiments, PCR was performed using dNTP (nondigoxigenin-labeled) for c-fos. The PCR product was electrophoresed on agarose gel and stained with ethidium bromide to detect amplified fragments. The PCR product was then identified by its size.

Sequencing

Each PCR product was purified after agarose gel electrophoresis with SUPEC-01 (TaKaRa, Tokyo, Japan), blunted by T4 polymerase, and subcloned into the EcoRV site of the Bluescript II KS (+) plasmid. The products were sequenced by the GENESIS 2000 DNA analysis system (Du Pont Medical Products, Wilminton, DE, USA) to confirm that they were the same as the published cDNA clones.

In situ hybridization

Hybridization was performed as described below. Sections were deparaffinized in xylene, dehydrated through ethanol, and air-dried. Basic protein was removed by incubation for 15min in 0.2N HCl at room temperature. After rinsing in 2 × SSC (20 × SSC is equivalent to 3M NaCl and 0.3M sodium citrate, pH 7.0) at room temperature, the sections were treated with 10mg/ml proteinase K (Amresco, OH, USA) in 20mM Tris-HCl pH 7.4 and 2mM CaCl$_2$ at 37°C for 1h, rinsed in phosphate-buffered saline (PBS), and refixed for 5min in 4% paraformaldehyde–PBS. The sections were then rinsed in PBS and acetylated for 10min in a freshly made 0.1M triethanolamine (pH 8.0) solution containing 0.25% acetic anhydride. The slides were washed three times in 2 × SSC for 5min, dehydrated through ethanol, and then air-dried.

The hybridization mixture contained 50% formamide, 5 × SSC, 5 × Denhardt's solution (100 × Denhardt's solution is equivalent to 2% Ficoll, 2% polyvinylpyrolidone [PVP], and 2% bovine serum albumin [BSA]), 0.1% SDS, 100mg/ml sonicated salmon sperm DNA (sssDNA), and 10% dextran sulfate. Each section was covered with 100ml hybridization solution containing 200ng DIG-labeled cDNA probe. Hybridization was carried out in a moist chamber at 37°C for 15h. The slides were subsequently washed twice in 2 × SSC at room temperature for 30min, and then washed twice with 50% formamide over 1 × SSC at 45°C for 10min. The colors of the sections were then developed with 5mM levamizole according to the manufacture's instructions (Boehringer Mannheim, Germany). Negative control experiments were performed either by pretreating tissue sections with RNase A (100μg/ml in 2 × SSC) at 37°C for 1h before hybridization, or by incubating tissue sections with an excess amount of unlabeled cDNA.

Immunohistochemistry

For the immunohistological analysis, the interfacial membranes were placed in OCT compound (Lab-Tek Products, Naperville, IL, USA) and stored at −70°C until cut. Sections were cut (5μm thick) on a cryostat, dried overnight, and fixed in cold acetone (4°C) for 10min. The fixed sections were incubated with 0.3% hydrogen peroxide for 10min to block endogenous peroxidase activity, and then treated with normal goat serum for 30min before incubation with the first antibodies. Rabbit polyclonal antibody to proto-oncogene c-fos (CALBIOCHEM, La Jolla, CA, USA) was diluted appropriately in phosphate buffer (pH 7.4, 140mmol/NaCl) and used as the first antibody. After incubation with the first antibodies for 45min, the section was exposed to biotinylated antirabbit immunoglobulin for 45min and then to avidin–biotin–peroxidase complex (Vectastain, Burlingame, CA, USA) for 30min. Finally, the site of peroxidase binding was disclosed with 3,3-diaminobenzidine tetrahydrochloride (Accurate, Westbury, New York, USA). The sections then were counterstained with hematoxylin. Between each step, the slides were washed three times for 5min each with PBS. Controls were performed as described, except for the omission of the first antibodies. Tartrate-resistant acid phosphatase (TRAP) staining and nonspecific elastase (NSE) staining were performed to identify osteoclasts.

Results

An inflammatory infiltrate was found, as well as numerous fibroblast-like cells. Higher magnification images of the results obtained by in situ hybridization are shown in Fig. 2a,b. There were c-fos-positive cells that lacked visible wear particles. C-fos mRNA was found in the cytoplasm of macrophage-like cells (Fig. 2a, indicated by a short arrow), fibroblast-like cells (Fig. 2a, indicated by an arrow head), and multinucleated giant cells (Fig. 2a, indicated by a long arrow), and as differentiation to osteoclast-like multinucleated cells progressed, the level of expression of the c-fos proto-oncogene decreased. Control staining using sense mRNA is shown in Fig. 2b.

In the immunohistological study, the localization of c-fos protein was examined. High levels of expression were found in the cytoplasm of macrophage-like cells (Fig. 3a), fibroblast-like cells (Fig. 3b), and multinucleated giant cells (Fig. 3c), and the level of expression of the c-fos proto-oncogene decreased as differentiation to osteoclast-like multinucle-

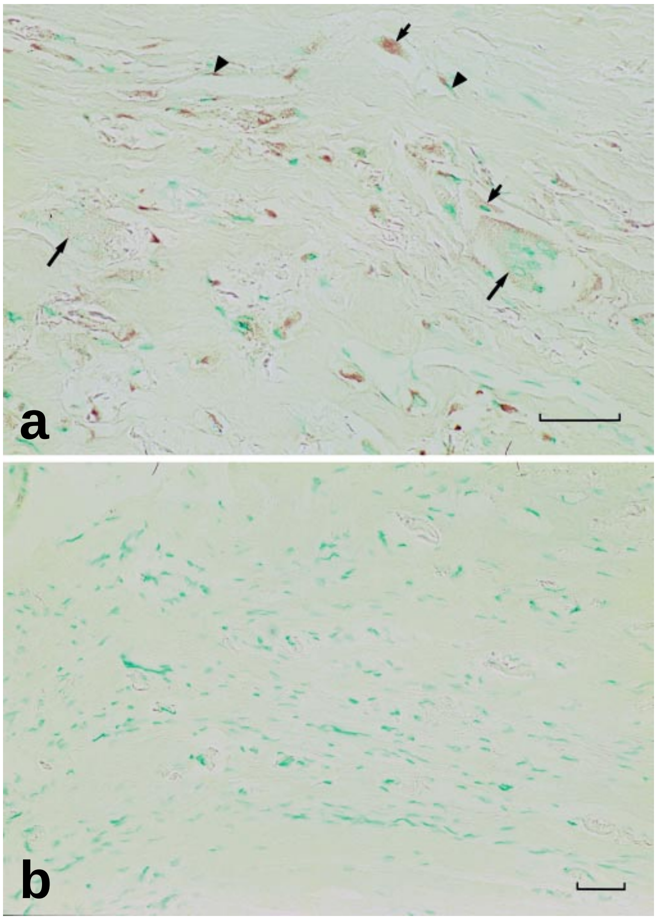

Fig. 2. High magnification images of c-fos mRNA expression, identified by in situ hybridization. **a** Macrophage-like cells (*short arrows*), fibroblast-like cells (*arrow heads*), and multinucleated giant cells (*long arrows*). **b** Control staining using sense mRNA. *Arrows* indicate areas of particulary intense c-fos expression. *Bar* 100μm

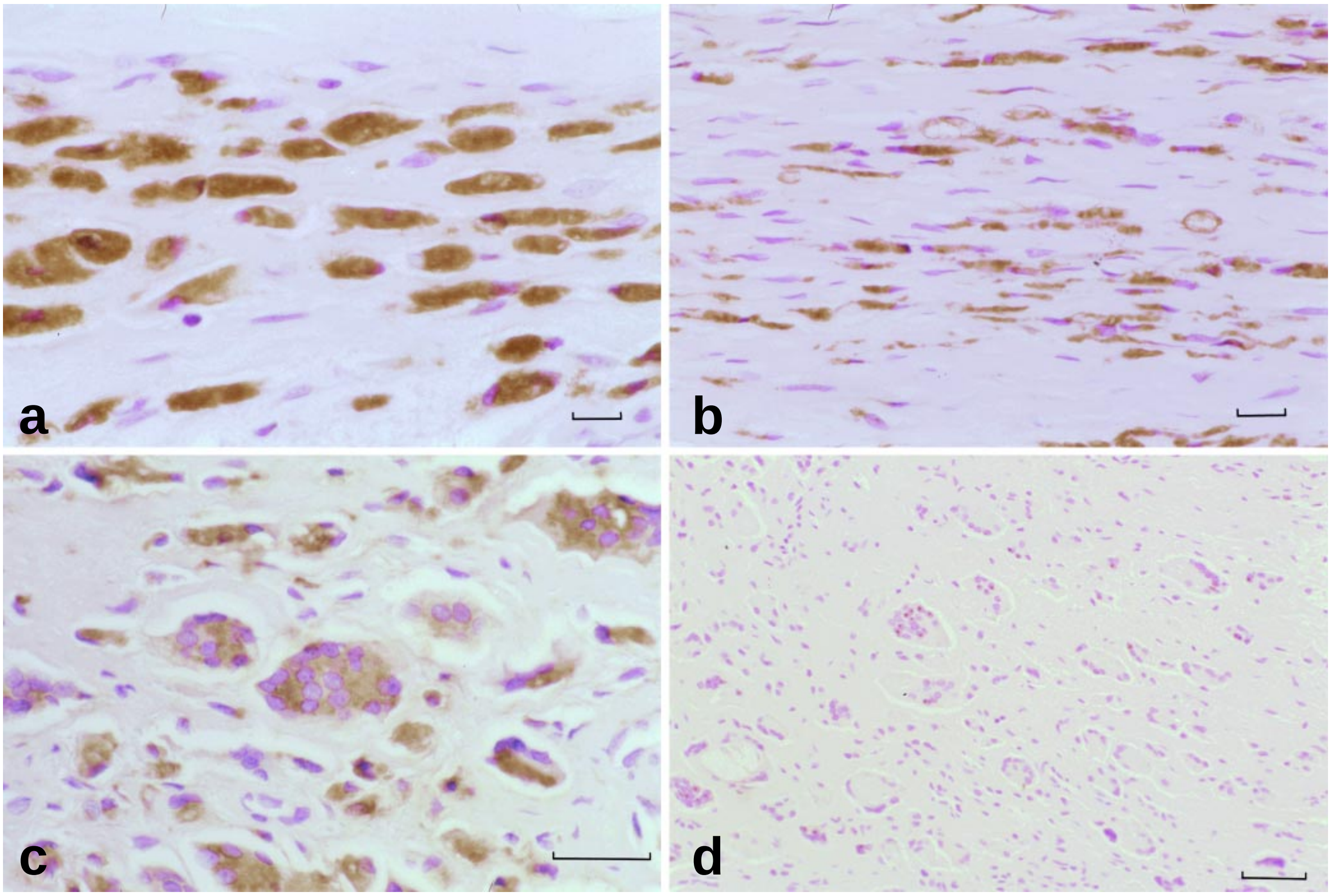

Fig. 3. Localization of c-fos protein (brown reaction product in the cytoplasm) in the interfacial membrane. **a** Macrophage-like cells, **b** fibroblast-like cells, *bar* 10 µm, **c** multinucleated giant cells, *bar* 100 µm, **d** negative control. *bar* 100 µm

ated cells progressed. Controls are shown as described, except for the omission of the first antibodies (Fig. 3d). Histological examinations of the tissue sections failed to identify the cells responsible for phagocytosis of the polyethylene wear particles and c-fos expression. No staining for c-fos by in situ hybridization or by immunohistochemistry was seen in normal human synovium (data not shown). Most of the cells with distinct cell bodies, such as macrophage-like cells and many multinucleated giant cells, were TRAP- and NSE-positive. These results suggest that precusors of osteoclasts are present in interface tissues, together with macrophage-like cells and multinucleated giant cells.

Discussion

These data confirm c-fos expression by human macrophage-like cells, fibroblast-like cells, and multinucleated giant cells in vivo, and they raise the possibility that c-fos is involved in aseptic loosening. Many of the macrophage-like cells that stained positively for c-fos may contain polyethylene particles. Maloney et al.[12] have reported that the mean size of polyethylene wear particles in periprosthetic membranes is 0.5 µm, which means that most of the particles are too small to be seen by optical microscopy.

There are two mechanisms involved in the development of rheumatoid arthritis: antigen-specific progression is followed by the progression of osteoclasia caused by proteolytic enzymes derived from the inflamed synovial membrane and the infiltration of pannus, which is an extension of inflammatory granulation tissue arising from the synovial membrane.[4] Expression of the c-fos gene in inflamed synovial membranes becomes important because the pannus continues to infiltrate over a long period. This is because it is granulation tissue which is susceptible to fibrosis, i.e., easily inactivated.[4,13] Therefore, when cells are stimulated through IL-1 receptors and TNF-α receptors, signal transduction via protein kinase C is activated, information is transmitted to the nucleus, and the production of stromelysin or collagenase increases via AP-1 of c-fos/c-jun.[14–17] In the interfacial membranes, wear debris may act as a trigger which activates macrophages or fibroblasts, and c-fos gene expression is up-regulated.

Osteoblasts synthesize bone matrix proteins such as collagen, and osteogenesis occurs when these are calcified. Osteoclasts maintain a balance between bone resorption and osteogenesis through activation by osteoblast-derived humoral factors, leading to bone resorption. Osteosarcoma and chondrosarcoma occur in transgenic mice with high expression of the c-fos gene.[6,7,18] Kuroki et al.[19] reported that when the c-fos gene is inserted in osteoblasts and its expression is high, the synthesis of collagen is inhibited, the induction of differentiation to osteoclast-like cells is promoted, and the formation of resorption cavities is increased. Recently, Hoyland and Sharpe[20] reported that c-fos gene expression is abnormally high in osteoclasts from patients with Paget's disease, and that this plays an important role in bone resorption.

However, when Grigoriadis et al.[6] compared c-fos knockout mice and normal mice, they found that macrophages were present in an inverse relationship to the decline of osteoclasts around trabecular bone in the long bones, and that the formation of osteoclasts was hindered by a lack of c-fos in blood cells. Therefore, it seems clear that c-fos is an important nuclear protein in apportioning the differentiation of macrophages and osteoclasts. In the present study, we showed that the c-fos gene and protein were not strongly expressed in typical multinucleated cells of osteoclasts. This finding suggests that proto-oncogene c-fos is not involved in mature osteoclast activation, and may affect the osteoclastic lineage of early stages of differentiation in vivo. Osteoclasts are TRAP-positive, but negative for NSE. The fact that multinucleated giant cells present in the membranous tissue were both TRAP- and NSE-positive suggests that these cells are osteoclast precusor cells. Although c-fos has already been implicated in rheumatoid arthritis, osteosarcoma, and Paget's disease, it has not previously been considered a factor in aseptic loosening.

In conclusion, these findings suggest the possibility that the c-fos gene and protein are deeply involved in the persistent chronic inflammation and osteolysis found around loosened hip prostheses.

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